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## **1. INTRODUCTION:**

The work we are conducting is aimed at understanding, and eventually preventing and treating, kidney disease, in particular the APOL1-associated form of kidney disease that accounts for the high rate of kidney disease in African Americans. This work is based on the hypothesis that APOL1 kidney disease in African Americans results from abnormal aggregation of the APOL1 risk variant protein in an amyloid-like process. We are testing this hypothesis in in vitro systems, cells, model organisms, and human kidney biopsy specimens using molecular biology, biochemistry, protein chemistry, and microscopy-based approaches.

## **2. KEYWORDS:**

Kidney, ESRD, APOL1, African American, FSGS, genetic

## **3. ACCOMPLISHMENTS:**

**What were the major goals of the project?**

### **1: Characterize APOL1 toxic oligomer formation in cell-free and cell-based systems**

We will determine whether risk variant APOL1 oligomer formation causes death of HEK293 cells by correlating the appearance of aggregates with cytotoxicity, confirming that intracellular A11 aggregates contain ApoL1 protein, and testing whether aggregation inhibitors or clearance of aggregates prevents cell death. Then we will compare the oligomerization process in vitro between WT and risk variant APOL1 to begin understanding how the aggregation process occurs.

### **2: Define the functional domains that govern APOL1 multimerization**

We will further refine our understanding of the aggregation process in risk variant APOL1 by characterizing the relative affinities of different ApoL1 variants for each other. We will map first the domains and then the specific amino acids that permit APOL1-APOL1 binding. We will use this information about sites of APOL1-APOL1 binding to design and test peptide aggregation inhibitors.

### **3: Compare the propensity of wild-type and risk mutant APOL1 to form toxic oligomers in model systems and human kidney biopsies**

We will test for risk variant APOL1 oligomer formation in model systems of increasing complexity. We will first simulate viral infection in human cells of different APOL1 genotype with interferon stimulation, then look for oligomer formation in our APOL1 transgenic zebrafish and mouse models, and lastly test human kidney biopsy samples of different APOL1 genotype and disease phenotypes for APOL1 oligomers. This aim will directly connect APOL1 oligomers with human kidney disease and also validate the use of these animal models for in vivo testing of new therapies.

## **What was accomplished under these goals?**

This update covers the 8-month period from our amended year 1 annual report submission (2/25/2016) to the present (10/25/2016).

### **Accomplishments/Activities**

#### **Overview:**

Over the past 8 months we have significantly extended our progress on the initial Aims of the grant. Though the Aims remain the same, some methodical approaches have changed. As described in our last progress report, we encountered large variation in the performance of oligomer specific antibodies (A11) from commercial vendors. In the interim period, we obtained additional lots of antibody from the academic laboratory at UCLA that discovered this class of antibodies. Work with these antibodies is described below. We have developed several alternative approaches addressing the same fundamental questions, as outlined in last year's report and with progress updates below.

#### Studies involving A11 anti-oligomer antibodies

##### Subtask 1.1A1.

Perform a time course study of APOL1 expressing cells (at 4, 8, 12, 16, 24, and 48 hours) and examine

- a. aggregate formation
- b. cell death

Subtask 1.1A3. Performs assays of cell lysates to look at aggregate formation using anti-A11 antibody

- a. dot blot assays
- b. non-denaturing Western blots

Subtask 1.1A4. Perform additional cell staining with organellar markers

- a. Identify subcellular localization of aggregates

Subtask 1.1A5. Perform immunoprecipitation

- a. precipitate A11 positive structures

A11 oligomer specific antibodies recognize the Beta sheet backbone structure common to amyloid conformations of multiple amyloid-forming proteins independent of amino acid side chains. Preliminary data using original lots of antibody indicated that APOL1 risk variants G1 and G2 expression in cells led to enhanced A11 staining in a spiculated pattern compared with the G0 (WT) APOL1 sequence. Experiments from year 1 of the grant period were performed with new lots of antibody from several commercial vendors (Millipore, Invitrogen); these demonstrated large amounts of background staining, obscuring the difference in putative amyloid or pre-fibrillar oligomer formation between APOL1 variants. Numerous technical modifications improved but did not entirely fix this problem. As a next step, this year we obtained new lots of A11 anti-oligomer antibody from the Glabe lab at UCLA that discovered A11 and other anti-

amyloid antibodies. We performed multiple dot-blot and immunofluorescence experiments attempting to replicate differences in A11 positivity between WT and risk variant APOL1 protein. Figure 1 shows a representative dot blot and figure 2 shows representative imaging by confocal microscopy. Background staining even in cells not transfected with APOL1-expressing plasmids demonstrates relatively strong positivity for A11 in dot blot analysis but much less by immunofluorescence. We will continue to work toward reducing the background staining from these new antibodies. We will also try the experiments in other cell types that adhere better to matrix-coated glass coverslips than HEK293 cells (such as HeLa cells) since we may be losing cells that become sick due to aggregate formation.

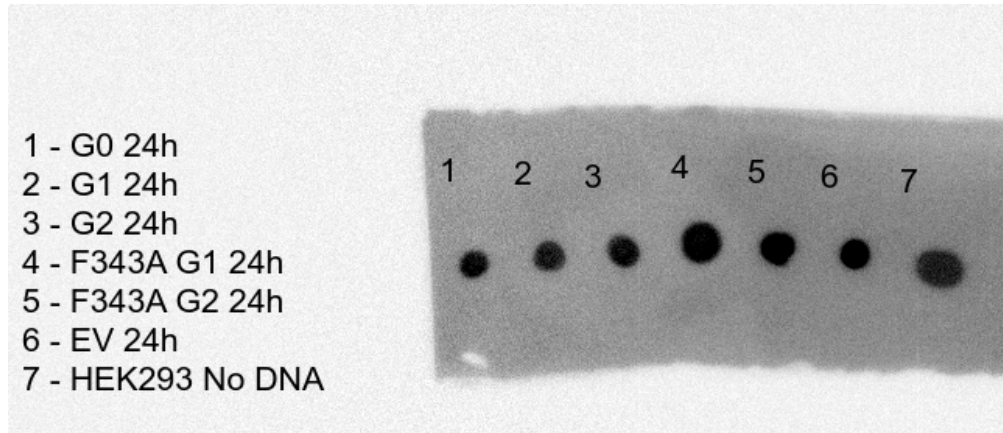


Figure 1. Dot blot with A11 anti-oligomer antibody. Lysates were prepared from HEK293 cells transfected with the indicated APOL1 variant expressing plasmid. Strong staining is seen even in cells not transfected with APOL1-expressing plasmid. This figure shows the Millipore antibody but antibodies from other sources performed similarly.

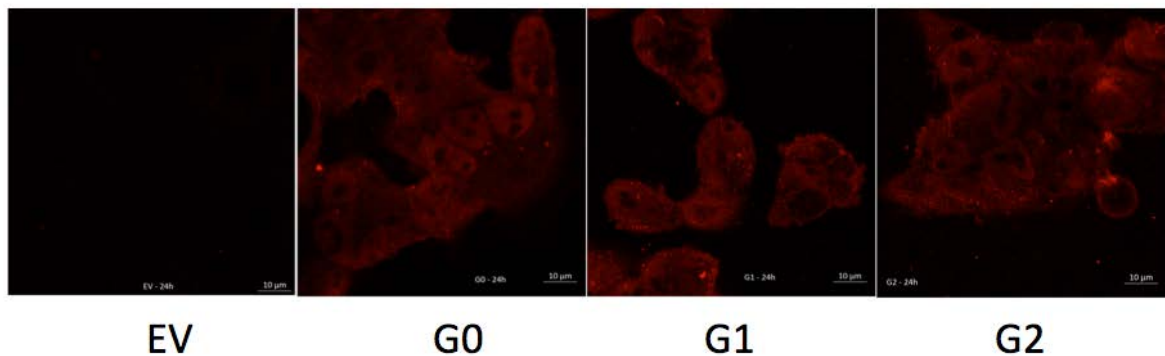


Figure 2. A11 anti-oligomer staining of HEK293 cells transfected with either empty vector, G0, G1, or G2 expressing APOL1 plasmids. A11 spicules appear stronger in G1 and G2 expressing cells on closer inspection, while diffuse staining is present in all APOL1 transfected cells.

Subtask 1.1B1: Test effect of compounds that block toxic oligomer formation on  
a. aggregate formation  
b. cell death

We extended our findings about the role of amyloid-inhibiting compounds such as thioflavin T and Congo Red dyes, and blockers of amyloid pores such as zinc ions. Previously demonstrated findings in tet-inducible stable APOL1-expressing cells (year 1 report, figure 3) was investigated in transiently transfected cells where we have the ability to rapidly modify amino acid sequence of the APOL1 protein through mutagenesis (Figure 3).

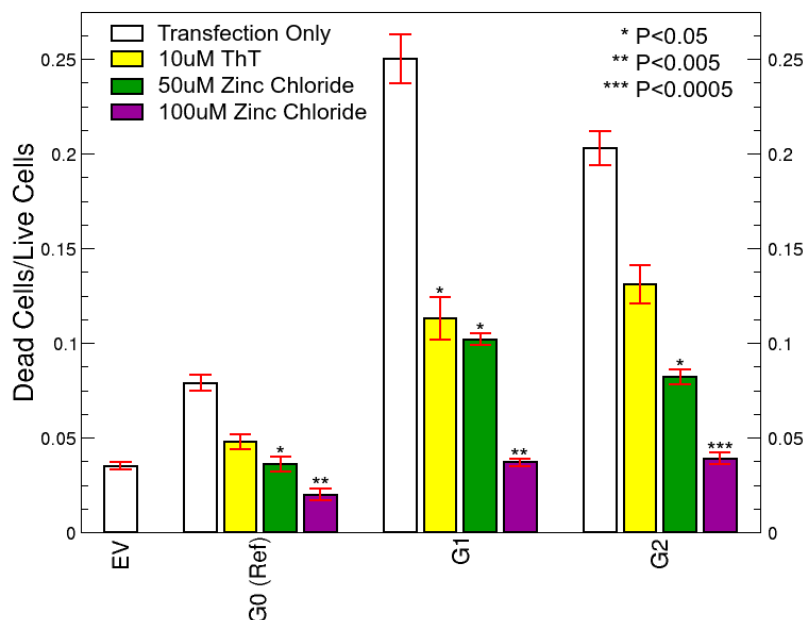


Figure 3. HEK293 cells were transiently transfected with plasmids expressing APOL1 WT (G0) or the APOL1 kidney risk variants G1 and G2. Thioflavin T (ThT) and zinc both block APOL1 induced toxicity. These are among the characteristics are typical of amyloid pores, discussed below.

Subtask 1.1B2. Test role of autophagy in aggregate formation and clearance  
a. Measure (1) aggregate formation and (2) cell death after autophagy gene knockdown in cells

While we found that inducers of autophagy such as rapamycin had a modest effect on cell death, knockdown of key cell death proteins such as ATG5, ATG7, and Beclin did not have large effects on APOL1 induced cell death in our experimental systems. We subsequently explored the other major pathway for removing aberrantly folded proteins, the proteasome system, to determine if APOL1 aggregates might be degraded by the proteasome or some combination of the proteasome and autophagy.

## 293 - 20h Transfection, 16h DMSO/250nM PS341

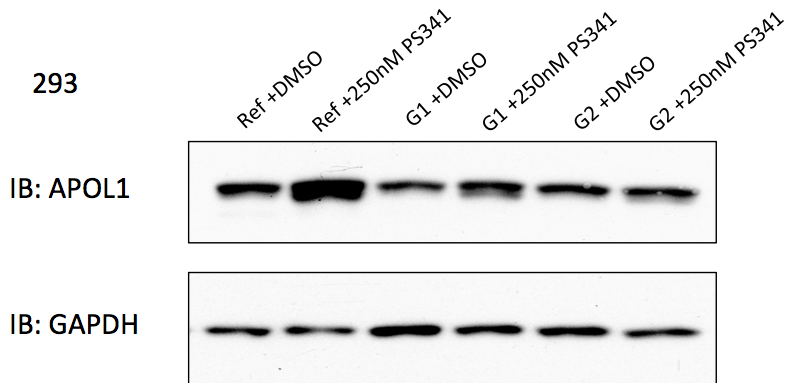


Figure 4. HEK293 cells were transfected with plasmids expressing APOL1 WT or risk variants (G1, G2). Cells were treated with the proteasome inhibitor PS341. As seen above, WT APOL1 accumulates in the presence of inhibitor whereas G1 and G2 accumulate much less. We interpret this data to show that WT APOL1 remains as small molecules amenable to proteasome degradation, whereas risk variants form higher order complexes that are degraded by other pathways.

### Subtask 2.1C

Perform Western blots using non-denaturing gels

Use combinations of ApoL1 holoprotein allelic variants and:

- look for differences in the size of the bands that may reflect monomers, dimers, trimers, or higher order oligomeric complexes.

We combined this subtask with new data from last year showing that mutation of amyloidogenic amino acid regions could eliminate or reduce cell death. We have performed initial experiments comparing the propensity of WT, G1, G2, or many different single amino acid substitutions to aggregate in cells as detected in non-denaturing gels and Western blotting. We continue to refine this technique.

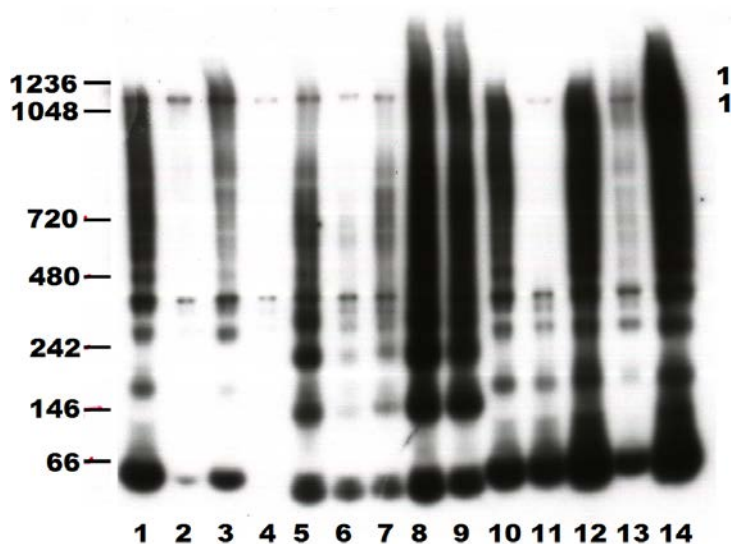


Figure 5. Native (non-denaturing) gels for different APOL1 variants. We are able to detect APOL1 in the monomer state (lowest band at around 44kd) but also many higher order structures. While this is a pilot attempt at this assay, we are looking for correlation between oligomerization behavior and APOL1 cytotoxicity. One technical obstacle we are working to overcome is enhanced cell death in risk variant expressing cells leads to loss of APOL1 staining because cells are lysing. We are testing earlier time points when less cell death



Subtask 1.2A: Please note that for each of the following subtasks, we will in general examine:

- in vitro translated ApoL1 protein
- purified full length protein
- synthesized peptide fragments.

Subtask 1.2A1. Characterize APOL1 in solution for light absorbance in presence of Thioflavin T

- Test both (1) APOL1 holoprotein and (2) amyloidogenic ApoL1 fragments
- Repeat, varying concentration, time, pH, and temperature.
- Compare WT with G1, G2 risk variants
- Perform assays with mixtures of WT, G1, G2.

We have generated peptide fragments from the amyloidogenic regions of APOL1 and tested them in Thioflavin T light absorbance assays to assess for amyloid formation. Shorter peptide fragments corresponding to the minimal amyloidogenic region (e.g. amino acids 339-353) were too hydrophobic to solubilize. Subsequently we used longer sequences that contained several hydrophilic amino acids with charged side chains to help solubilize the fragments, which proved somewhat effective. Our early experiments suggest differences in light absorbance between WT and risk variant APOL1, but not in ways that were anticipated. However, a collaborator has developed a new protocol for solubilizing APOL1 protein from inclusion body aggregates made by *E. coli*, so we will attempt these experiments with holoprotein instead of fragments, as the holoprotein is the biologically relevant molecule.

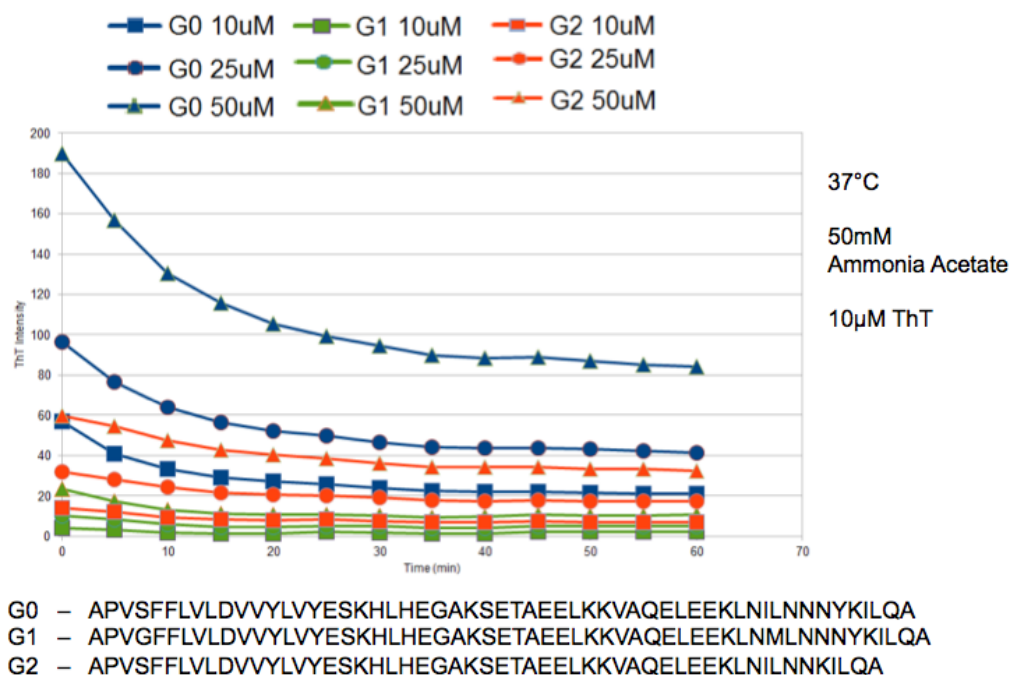


Figure 6. This figure represents pilot studies looking for differential ThioflavinT (ThT) binding to APOL1 peptide fragments containing the APOL1 amyloidogenic sequence. The x axis shows time in solution and the y axis shows intensity of ThT light absorbance in the spectrophotometer. These studies are limited by the strong hydrophobicity of the amyloidogenic region, making the fragments difficult to get into solution. We plan to continue these studies using holoprotein, which can now be isolated from inclusion bodies produced in bacteria.

### Additional subtasks introduced in progress report #1.

We made significant progress identifying key amyloidogenic amino acids, as demonstrated in figure 11 of last year's progress report. We showed that a phenylalanine located directly next to the G1 substitution S342G was essential for toxicity, and substitution of that amino acid by all but planar aromatic amino acids mitigated cell death. The predicted direct apposition of the G1 and G2 mutations in a coiled coil domain suggested the hypothesis that the aromatic rings at amino acid 343 might be interacting with the planar, aromatic tyrosine at a.a. 389. Substitution experiments near the G2 mutation (a.a. 389) did not bear this out, but we have now mapped the amino acids essential for cell death. Together with structural information we discovered about the APOL1 C-terminus using NMR and molecular simulations, our mutagenesis studies have defined the amino acids necessary for APOL1 induced cell death and predicted effects of these mutations on C-terminal structure (data not shown).

Our experiments suggest that APOL1 could be causing two different kinds of cell death: one induced by amyloid aggregates and another induced by APOL1 amyloid pores. Amyloid pore activity is supported by 1) amyloid structure predictions, 2) acid requirement for membrane insertion, 3) inhibition by amyloid binding dyes and zinc, and 4) cation flux in membranes.

Amyloid pore activity would explain one of the fundamental mysteries of APOL1 kidney disease, namely why the disease appears to be inherited in a recessive fashion despite behaving like a gain-of-function mutation. This scenario is best explained by a multi-subunit APOL1 oligomer, with wild type APOL1 able to rescue the toxicity of mutant APOL1.

To begin exploring this possibility, we have attempted several rescue experiments to determine if WT APOL1 can rescue the toxicity of risk variant APOL1. This type of rescue would substantiate, albeit indirectly, that APOL1 forms oligomers and that oligomers that contain WT APOL1 are rendered non-toxic. Our preliminary data is shown below (figure 7):

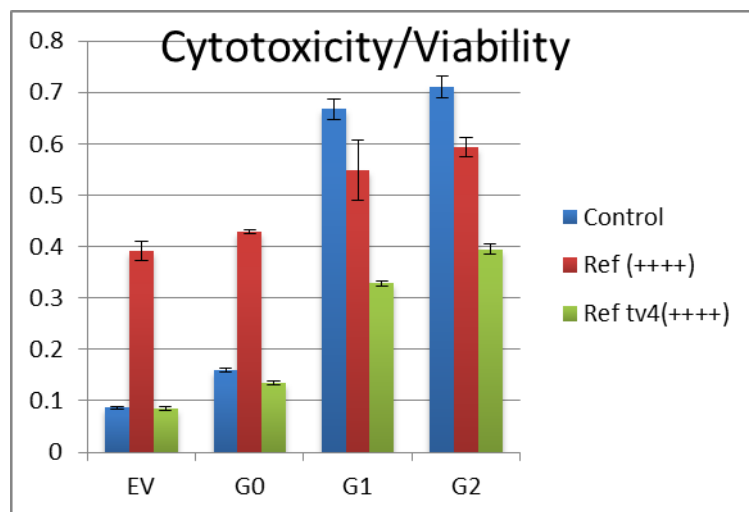


Figure 7. Rescue of risk variant (G1, G2) induced cell death by WT APOL1 would strongly support direct APOL1-APOL1 interaction, explaining why humans heterozygotes for the APOL1 risk variants do not develop disease. Our early experiments do not show rescue at 1:1 transfection of risk variant and WT APOL1, but excess WT APOL1 (at a 4:1 or 10:1 ratio, shown here) does promote rescue. However, several important control experiments need to be performed to rule out alternative causes of rescue (such as altered expression levels of the risk variants).

**What opportunities for training and professional development has the project provided?**

Nothing to Report.

**How were the results disseminated to communities of interest?**

Drs. Friedman and Pollak have presented aspects of this work at several meetings related to kidney disease.

**What do you plan to do during the next reporting period to accomplish the goals?**

1. (Friedman and Pollak labs) Characterize APOL1 oligomerization state in solution using holoprotein and light scattering measurements.
  - a. Compare WT, G1, and G2 oligomerization tendencies
  - b. Test point mutations in predicted amyloidogenic amino acids
  - c. Performing mixing assays to determine whether WT or non-toxic point mutants can alter the oligomerization dynamics of risk variants.
2. (Friedman Lab) Continue to study oligomerization states using native (non-denaturing) gel electrophoresis.
  - a. Test WT, G1, and G2 characteristics, and characteristics of point mutants that alter APOL1 cytotoxicity
  - b. Perform mixing experiments to test whether APOL1 variants not prone to oligomerization can prevent oligomerization of toxic APOL1 variants.
3. (Pollak Lab) Use cell fractionation techniques to determine whether oligomers become insoluble or traffic to certain cellular compartments.
4. (Friedman Lab) Further explore the N-terminal predicted amyloidogenic domain to complement the detailed work we have done dissecting the C-terminal predicted amyloidogenic domain.
5. (Pollak Lab) Test A11 staining in APOL1 transgenic mouse kidneys. These mouse models have been developed for other projects but tissue from mice used in these other projects will be used to look for evidence of toxic oligomer (A11 positive) staining.

**4. IMPACT:**

**What was the impact on the development of the principal discipline(s) of the project?**

The potential impact of this project is to completely change the way investigators think about the mechanism of APOL1 kidney disease. Success here will have immediate and substantial implications for therapy for APOL1 kidney disease. We have not yet demonstrated that our hypothesis is correct, but we remain committed to testing the toxic oligomerization hypothesis.

**What was the impact on other disciplines?**

Nothing to report

**What was the impact on technology transfer?**

Nothing to report

**What was the impact on society beyond science and technology?**

Nothing to report

## **5. CHANGES/PROBLEMS:**

**Changes in approach and reasons for change**

Problems with a key reagent, the A11 anti-oligomer antibody, have led to alternative approaches that are proving quite successful. Both the problems and the alternative approaches are summarized in the progress report under “accomplishments.”

**Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to report

**Changes that had a significant impact on expenditures**

Nothing to report

**Significant changes in use or care of human subjects**

Nothing to report

**Significant changes in use or care of vertebrate animals**

Nothing to report

**Significant changes in use of biohazards and/or select agents**

Nothing to report

## **6. PRODUCTS:**

- **Publications, conference papers, and presentations**

**Journal publications**

Manuscript in preparation

**Books or other non-periodical, one-time publications.**

Nothing to report

**Other publications, conference papers and presentations.**

Nothing to report

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

New DNA clones developed during the work on this project will be shared with the scientific community per request after publication.

- **Inventions, patent applications, and/or licenses**  
Nothing to report
- **Other Products**  
Nothing to report

## 7. PARTICIPANTS

**What individuals have worked on the project?**

Name:	David J. Friedman
Project Role:	Initiating PI
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person months worked:	3.0
Contribution to Project:	As PI, overseeing all aspects of the project
Funding Support:	none

Name:	Herbert Lannon
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person months worked:	12.0
Contribution to Project:	Conducting experiments to look at amyloidogenic properties of APOL1
Funding Support:	NIDDK T32

Name:	Shrijal Shah
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	n/a
Nearest person months worked:	8.0
Contribution to Project:	Conducting experiments to examine effects of inflammatory stimuli on APOL1 interactions
Funding Support:	

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

NephCure Kidney International Grant ended.  
Vertex Sponsored Research Agreement started.

Vertex (no number) (PI: Pollak and Friedman) 07/07/2016 - 07/07/2019  
Title: Studies on APOL1 Kidney Disease \$954,049 3.0 CM  
Goal: To develop tools to better understand and treat APOL1-associated kidney disease  
Overlap: None.  
Grant officer: ATTN: Contracts Management, Vertex Pharmaceuticals, Inc.  
50 Northern Ave., Boston, MA

**What other organizations were involved as partners?**

Nothing to Report